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(54) LIVE VACCINES

LEBENDE VAKZINE

VACCINS VIVANTS

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Description

The present invention relates to attenuated microorganisms, to their use in the immunoprophylaxis of an animal or a human, and to vaccines containing them.

The principle behind vaccination or immunoprophylaxis is to induce an immune response in an animal to a pathogenic organism by innoculation with an attenuated strain of the organism thus providing protection against subsequent challenge. In 1950 Bacon et al (Br.J.Exp.Path. 31, 714-724) demonstrated that certain auxotrophic mutants of S.typhi were attenuated in mice when compared to the parental strain. Certain of these auxotrophic mutants have been proposed as being suitable candidates for the basis of a whole cell vaccine. (See for example Hosieth and Stocker; Nature, 1981 241, 238-239, and European patent publication 322,237). In addition to mutations in an essential auxotrophic pathway, other loci have been identified where mutations result in attenuation of microorganisms. Examples of such loci include regulons that exert pleiotrophic effects, e.g., the cya/crp system (Roy Curtiss III et al, Vaccine 6, 155-160, 1988) and the ompR envZ system (Dorman et al, Infect.Immun. 57, 2136-2140, 1989) and the phoP system (Fields et al, Science 243, 1059-1062, 1989).

In many microorganisms, between one and two dozen proteins are produced in response to a range of different environmental stresses, such as high temperature, nutrient deprivation, toxic oxygen radicals and metabolic disruption. These represent part of the coordinated regulation of various different genes induced in response to the particular stress to which the microorganism is subjected. The family of major stress proteins (also known as heat shock proteins) is amongst the most highly conserved in nature. Substantial homology exists amongst members of this family isolated from E.coli, Drosophilia spp. and man (for a recent review see Neidhardt, G.C. & Van Bogelen, R.A. (1987) Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology. F.C. Neidhardt et al. eds. pp. 1334-1345. American Society for Microbiology, Washington DC). For example: Hsp90, Hsp70 and Hsp60 are heat shock proteins found in all prokaryotes and eukaryotes. Amino acid sequence comparison between Hsp90 from E.coli and that from man shows that approximately half the amino acid residues are identical. Other members of the stress protein family are GrpE, GroEL, DnaK, CroES, Lon and DnaJ.

The genes encoding the family of heat shock proteins are transcribed by RNA polymerase co-operating with the σ^{32} factor, the product of the rpoH gene (reviewed by Neidhardt, F.C. and van Bogelen, R.A, 1987. In Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Neidhardt, F.C. et al eds. pp. 1334-1345, American Society for Microbiology, Washington, D.C.). Recently, Lipinska et al (Nucleic.Acids.Res. 1988 21, 10053-10067) have described a heat shock protein in E.coli, referred to as HtrA, that appears to be σ^{32}-independent. Examination of the promoter region of the htrA gene shows DNA sequence homology with the P.3 promoter of the rpoH gene; a promoter known to be recognised by $\sigma^{E}(\sigma^{24})$ factor. This similarity suggests that the htrA promoter may also be recognised by the RNA polymerase-$\sigma^{E}(\sigma^{24})$holoenzyme.

Phenotypically, in E.coli, a mutation in the htrA locus abolishes the ability of bacterium to survive at temperatures above 42°C (Lipinska et al, 1989, J.Bacteriol, 171, 1574-1584). The gene maps at 4 min on the E.coli chromosome and encodes a protein with a relative mulecular mass (Mr) of 51,163. This protein precursor undergoes N-terminal processing involving the removal of a signal peptide sequence (Lipinska et al, 1988, Nucleic.Acids.Res. 21, 10053-10067), to yield the mature form of the polypeptide upon secretion through the inner membrane of the bacterium. Independently, the htrA gene has been identified as degP by Strauch, K.L. and Beckwith, J. 1988 (Proc.Natl.Acad.Sci. USA 85, 1576-1580) who were examining E.coli mutants with decreased protease activity, degP mutants were isolated by TnphoA mutagenesis (Manoil, C. & Beckwith, J. 1985, Proc.Natl.Acad.Sci. USA 82, 8129-8133) and were recognised by the increased stability of a hybrid Tsr-phoA (Tsr-AP2) recombinant protein in a degP background (Strauch, K.L. and Beckwith, J. 1988. Proc.Natl.Acad.Sci. USA 85, 1576-1680). In E.coli the genes identified as degP and htrA appear to be identical and encode a protein that is a member of the "stress-response" family.

The present invention provides a vaccine comprising a pharmaceutically acceptable carrier and an effective amount of a bacterium attenuated by a non-reverting mutation in the htrA gene. The invention also provides a bacterium attenuated by a non-reverting mutation in a the htrA gene for use in the prophylactic treatment of a host against infection by a microorganism.

The bactaria for use with the present invention are preferably bacteria, especially Gram-negative bacteria, which invade and grow within eucaryotic cells and colonise the mucosal surface. Examples of these include members of the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia. In particular the following species can be mentioned: S.typhi - the cause of human typhoid; S.typhimurium - the cause of salmonellosis in several animal species; S.enteritidis - a cause of food poisoning in humans; S.choleraesuis - the cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; and Neisseria gonorrhoeae - the cause of gonorrhoea. The invention includes a Salmonella bacterium attenuated by a non-reverting mutation in the htrA gene.

A non-reverting mutation is one which cannot be repaired in a single step. Genetic mutations of this sort include deletion, inversion, insertion and substitution mutations. Deletion mutations can be generated using transposons.

These are DNA sequences comprising from between 750 to thousands of base pairs which can integrate into the host's chromosomal DNA. The continuity of the DNA sequence of interest is thus disrupted with the loss of gene function. Transposons can be deleted from the host chromosomal DNA; most frequently excision is imprecise leading to a non-reverting mutation. Substitution or insertion mutations can arise by use of an inactivated DNA sequence carried on a vector which recombines with or crosses-over with the DNA sequence of interest in the host's chromosomal DNA with the consequent loss of gene function.

The sequence of the htrA gene is set out in Fig. 1. (SEQ ID No: 1) (also characterised as degP).

For use in the form of a live vaccine, it is clearly important that the attenuated bacterium used in the present invention does not revert back to the virulent state. The probability of this happening with a mutation in a single DNA sequence is considered to be small. However, the risk of reversion occurring with a bacterium attenuated by the presence of mutations is each of two discrete DNA sequences is considered to be insignificant. It is preferred therefore that attenuation of the bacterium is also brought about by the presence of a mutation in a second gene. The second gene preferably encodes an enzyme involved in an essential auxotrophic pathway or is a gene whose product controls the regulation of osmotically responsive genes, i.e. ompR (Infect and Immun 1989 2136-2140). Most preferably, the mutation is in a gene involved in the aromatic amino acid biosynthetic pathway, more particularly the genes encoding aroA, aroC or aroD (EP-A- 322237).

The bacteria used in the present invention are constructed by the introduction of a mutation into the DNA sequence by methods known to those skilled in the art (Maniatis, Molecular Cloning and Laboratory Manual, 1982). Non-reverting mutations can be generated by introducing a hybrid transposon TnphoA into, for example, S.typhimurium strains. TnphoA can generate enzymatically active protein fusions of alkaline phosphatase to periplasmic or membrane proteins. The TnphoA transposon carries a gene encoding kanamycin resistance. Transductants are selected that are kanamycin resistant by growing colonies on an appropriate selection medium.

Alternative methods include cloning the DNA sequence into a vector, e.g. a plasmid or cosmid, inserting a selectable marker gene into the cloned DNA sequence, resulting in its inactivation. A plasmid carrying the inactivated DNA sequence and a different selectable marker can be introduced into the bacterium by known techniques (Maniatis, Molecular Cloning and Laboratory Manual, 1982). It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the bacterium and the wild-type DNA sequence has been rendered non-functional in a process known as allelic exchange. In particular, the vector used is preferably unstable in the bacterium and will be spontaneously lost. The mutated DNA sequence on the plasmid and the wild-type DNA sequence may be exchanged by a genetic cross-over event. Additional methods eliminate the introduction of foreign DNA into vaccine strains at the site of mutations.

An attenuated bacterium may be produced by a process which comprises introduction of a mutation in the htrA gene by either

a) transposon mutagenesis; or

b) transforming the bacterium with a vector incorporating an htrA gene containing a non-reverting mutation, and screening to select the desired bacterium.

The attenuated bacterium optionally expresses a heterologous antigen. This expression is likely to be favourable in htrA mutants because of the increased stability of recombinant antigens associated with the degP phenotype. Such antigens may be viral, bacterial, protozoal or of higher parasitic microorganisms. Such bacteria may then form the basis of a bi- or multi-valent vaccine. Examples of useful antigens include E.coli heat labile toxin B subunit (LT-B), E. coli K88 antigens, FMDV (Foot and Mouth) peptides, Influenza viral proteins, P.69 protein from B.pertussis. Other antigens which could be usefully expressed would be those from Chlamydia, flukes, mycoplasma, roundworms, tapeworms, rabies virus and rotavirus.

A bacterium expressing DNA encoding a heterologous antigen may be produced by transformation of the microorganism with an expression cassette. Expression cassettes will include DNA sequences, in addition to that coding for the heterologous antigen, which will encode transcriptional and translational initiation and termination sequences. The expression cassette may also include regulatory sequences. Such expression cassettes are well known in the art and it is well within the ability of the skilled man to construct them. The expression cassette may form part of a vector construct or a naturally occurring plasmid. An example of a genetically engineered attenuated Salmonella which is capable of expressing a heterologous antigen is described in EP-A-127,153. The expression cassette may also be engineered to allow the incorporation of the heterologous gene into the chromosome of the bacterium.

A further bivalent vaccine comprising an attenuated Salmonella typhi, capable of expressing the E.coli heat-labile enterotoxin subunit B is disclosed by Clements et al (Infection and Immunity, 46, No.2. 1984, 564-569). Ty21a, an attenuated S.typhi strain, has been used to express other antigens such as the Shigella sonnei form I antigen (Formal et al., Infection and Immunity, 34, 746-750, 1981).

The vaccine of the invention is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising for example Eudragate "S" Eudragate "L" Cellulose acetate, cellulose pthalate or hydroxy propylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared by parenteral administration, intranasal administration or intramammary.

A host (particularly a human host) may be prophylactically treated against an infection caused by a microorganism by administering to said host an effective dose of a vaccine according to the invention. The dosage employed in such a method a treatment will be dependent on various clinical factors, including the size and weight of the host and the type of vaccine formulated. However, for attenuated S.typhi a dosage comprising the administration of 10^9 to 10^{11} S. typhi organisms per dose is generally convenient for a 70kg adult human host.

The following examples provide experimental details in accordance with the present invention. It will be understood that these examples are not intended to limit the invention in any way.

Figure Legend

Figure 1. DNA sequence of the htrA gene and the amino acid sequence of the protein it encodes.

Figure 2. Sensitivity of S.typhimurium htrA mutant 046 to temperatures above 42°C and oxygen radicals

Figure 3. In vivo kinetics of S.typhimurium strains harbouring a mutation in htrA (BRD726) and htrA aro mutations (BRD807).

Example 1

Identification of the htrA gene in Salmonella typhimurium and generation of an htrA mutant.

TnphoA mutagenesis was used in the mouse virulent Salmonella typhimurium strain C5 (Miller et al, 1989, Infect. Immunol, 57, 2758-2763). Mutants were selected likely to harbour lesions in genes that have a signal peptide sequence, i.e. proteins likely to be targeted through a bacterial membrane. Isolation of the DNA flanking the TnpboA insertion identifies the gene that has been insertionally activated. This gene was isolated and its DNA sequence was determined by standard methods (see Figure 1. SEQ ID No: 1) (Maniatis et al., 1982, In Molecular Cloning: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.; Sanger et al., 1977, Proc.Natl.Acad.Sci. USA 74, 5463-5467). Comparison of the translated protein sequence with sequences held in the EMBL Database showed surprisingly that it shared 88% homology with the sequence of the htrA product from E.coli (Fig.1. SEQ ID No: 1).

Example 2

Identification of hTrA in S.typhimurium as a gene involved in the stress-response

E.Coli mutants harbouring lesions in the htrA gene are unable to grow at temperatures above 42°C. The S.typhimurium htrA mutant, 046, was tested for growth at elevated temperatures and was found to grow as well as the present strain C5. However, when tested for sensitivity to oxygen radicals, the mutant 046 showed decreased resistance as compared with the parent C5 strain clearly indicating that the gene is responsible (at least in part) for this aspect of the stress response (see Fig. 2).

Example 3

Comparison of attenuated Salmonella typhimurium strain 046 with virulent parent strain Salmonella typhimurium C5.

The attenuated strains were constructed using TnphoA transposon mutagenesis as described previously (Miller et al., 1989, Infect. Immun. 57, 2758-2763).

After oral administration the mutant strain 046 had a Log_{10} LD_{50} of greater than 9 cells as compared to the parental strain, C5, which has a Log_{10} LD_{50} of 6.38 cells. (All ID_{50} were calculated after 28 days). Thus 046 is highly attenuated. After i.v. administration 046 had an i.v. Log_{10} LD_{50} of 5.13 cells compared to less than 10 cells for C5 and we again

conclude that 046 is highly attenuated compared to C5.

Example 4

Protection of mice after oral challenge.

Mice were immunised with 046 and challenged 28 days later with the virulent parental strain C5. Mice vaccinated with using 10^{10} cells of 046 showed excellent protection against challenge with C5. eleven weeks after vaccination. The Log_{10} LD_{50} in immunised animals was 9.64 cells compared with 6.6 cells for unimmunised controls. Thus, mice vaccinated orally with a single dose of 046 were well protected against virulent C5 challenge.

Example 5

Construction of a defined S.typhimurium SL1344 htrA mutant

Sequence data facilitated the identification of suitable restriction endonuclease sites that could be used to introduce a deletion into the htrA gene. A 1.2Kb deletion was introduced by digesting with EcoRV and religating. A drug resistant marker was also introduced into the gene (Kanamycin cassette, Pharmacia) by standard techniques to enable selection for the presence of the deleted gene. The plasmid harbouring the deleted htrA gene was introduced into a polA strain S.typhimurium (BRD207) in which the plasmid cannot replicate. The only way that kanamycin resistance can be maintained in the host is if there has been a recombination event between the S.typhimurium sequences on the vector and the homologous regions on the chromosome. Loss of ampicillin resistance while maintaining kanamycin resistance indicates a second homologous recombination event resulting in the replacement of the intact htrA gene with the deleted one. Colonies resistant to kanamycin were isolated and checked for ampicillin resistance. One colony that was kanamycin resistant and ampicillin sensitive was selected for further study and was designated BRD698 (deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No NCTC 12457 on 22 March 1991 in accordance with the terms of the Budapest Treaty).

A P22 lysate was prepared on this strain by standard techniques (Dougan et al, J.Infect.Dis. 158, 1329-1335, 1988) and used to infect SL1344. Kanamycin resistant colonies were isolated and checked for the presence of the deletion by Southern hybridisation. One strain, designated BRD726 (deposited at PHLS under Accession No. NCTC 12458 on 22 March 1991 in accordance with the terms of the Budapest Treaty) was selected for further study.

Example 6

Construction of an S.typhimurium SL1344 aroA htrA double mutant

The P22 lysate prepared on BRD698 was used to introduce the htrA deletion into an S.typhimurium SL1344 strain already harbouring a deletion in aroA. The method for introducing an aroA deletion has already been described by Dougan et al, J.Infect.Dis. 158, 1329-1335, 1988. One strain that was found to have deletions in both aroA and htrA was selected for further study and was designated BRD807, (deposited at PHLS under Accession No. NCTC 12459 on 22 March 1991 in accordance with the terms of the Budapest Treaty).

Example 7

Comparison of the attenuation of SL1344 htrA (BRD726) and SL1344 htrA and aroA (BRD807) with the virulent parent strain SL1344

After oral administration BRD726 and BRD807 had Log_{10} LD_{50}s of >10.0 cells compared to the virulent parent strain which has a Log_{10} LD_{50} of 6.8 cells*. Both strains were therefore highly attenuated compared to the virulent parent strain SL1344.

Example 8

Assessment of oral vaccine potential of BRD726 and BRD807

BALB/c mice were orally immunised with approximately 10^{10} cells of BRD726 and BRD807 as previously described (Dougan et al, J.Infect.Dis. 158, 1329-1335, 1988) and challenged 4 and 10 weeks later with the virulent parent strain SL1344. LD_{50}s were calculated by the method of Reed and Muench (Am.J.Hyg. 27, 493-497, 1934). All determinations

*all LD_{50}s were calculated after 28 days.

were carried out at least twice. Mice vaccinated with BRD726 and BRD807 showed excellent protection against challenge with SL1344 at 4 weeks, the $\log_{10}$ LD_{50}s being >10.0 and 9.7 cells respectively. This compares with log 6.1 cells for unimmunised controls. At 10 weeks $\log_{10}$ LD_{50}s for BRD726 and BRD807 were 9.11 and 8.11 cells compared to 6.5 for SL1344. Thus the mice immunised with BRD726 had excellent long term immunity to virulent SL1344 challenge. This compares favourably with protection elicited by double aro mutants of SL1344 (Dougan et al, J.Infect.Dis. 158, 1329-1335, 1988). The long term protection afforded by vaccination with BRD807 is 46-fold better than unimmunised controls. Thus both BRD726 and BRD807 make good vaccine strains for BALB/c mice.

Example 9

In vivo kinetics of BRD726 and BRD807 in BALB/c mice

The ability of BRD726 and BRD807 to grow in vivo after intravenous administration was assessed. Mice were infected with approximately 10^5 organisms. Numbers of bacteria in livers and spleens were enumerated at different times during the infection up to 21 days. The results obtained are shown in Fig3. Neither BRD726 or BRD807 underwent an initial period of replication in murine tissues. The strains are cleared slowly from the organs and by day 21 BRD807 has almost cleared from the murine tissues while BRD726 is still persisting at low levels.

Example 10

Formulation

An attenuated microorganism of the present invention is preferably presented in an oral tablet form.

INGREDIENT		MG/TABLET
Core tablets		
1.	Freeze-dried excipient carrier containing $10^9$$10^{10}$ attenuated bacteria.	70.0
2.	Silica dioxide (Aerosil 200)	0.5
3.	Dipac (97% sucrose)	235.0
4.	Cross-linked Povidone (Kollidon CL)	7.0
5.	Microcrystalline Cellulose (Avicel pH102)	35.0
6.	Magnesium Stearate	2.5
Coating		
7.	Opadry Enteric, OY-P-7156 (Polyvinyl acetate phthalate + Diethylphthate)	35.0
		385.0

A carrier containing 5% sucrose, 1% sodium glutamate and 1% bacto casitone in an aqueous solvent is prepared. The organisms are suspended in this carrier and then subjected to freeze-drying.

The freeze-dried material is blended with Aerosil 200 and the blended mixture is sifted through a screen. The sifted powder is mixed with Dipac, Kolidan CL, Aricel pH102 and Magnesium Stearate in a blender. This blend is compressed into tablets for subsequent enteric coatings.

The skilled man will appreciate that many of the ingredients in this formulation could be replaced by functionally equivalent pharmaceutically acceptable excipients.

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, SE

1. A vaccine comprising a pharmaceutically acceptable carrier and an effective amount of a bacterium attenuated by a non-reverting mutation in the htrA gene.

2. A vaccine as claimed in claim 1, wherein the mutation is a deletion or insertion mutation.

3. A vaccine as claimed in claim 1 or 2, wherein the bacterium is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.

4. A vaccine as claimed in claim 3, wherein the Salmonella bacterium is selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

5. A vaccine as claimed in any one of the preceding claims, in which attenuation of the bacterium is also brought about by a mutation in a second gene.

6. A vaccine as claimed in claim 5, wherein the mutation in a second gene is in a gene involved in the aromatic amino acid biosynthetic pathway.

7. A vaccine as claimed in claim 6, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

8. A vaccine as claimed in any one of the preceding claims, wherein the bacterium expresses DNA encoding a heterologous antigen.

9. A vaccine as claimed in any one of the preceding claims adapted for oral administration.

10. A bacterium attenuated by a non-reverting mutation in the htrA gene for use in the prophylactic treatment of a host against infection by a microorganism.

11. A bacterium as claimed in claim 10, which is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.

12. A Salmonella bacterium attenuated by a non-reverting mutation in the htrA gene.

13. A bacterium as claimed in claim 10, 11 or 12, wherein the mutation is a deletion or insertion mutation.

14. A bacterium as claimed in any one of claims 11 to 13 selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

15. A bacterium as claimed in any one of claims 10 to 14, in which attenuation is also brought about by a mutation in a second gene.

16. A bacterium as claimed in claim 15, wherein the mutation in a second gene is in a gene in the aromatic amino acid biosynthetic pathway.

17. A bacterium as claimed in claim 16, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

18. A bacterium as claimed in any one of claims 10 to 17 which expresses DNA encoding a heterologous antigen.

Claims for the following Contracting States : ES, GR

1. A method of producing a vaccine, comprising mixing a pharmaceutically acceptable carrier and an effective amount of a bacterium attenuated by a non-reverting mutation in the htrA gene.

2. A method as claimed in claim 1, wherein the mutation is a deletion or insertion mutation.

3. A method as claimed in claim 1 or 2, wherein the bacterium is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.

4. A method as claimed in claim 3, wherein the Salmonella bacterium is selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

5. A method as claimed in any one of the preceding claims, in which attenuation of the bacterium is also brought about by a mutation in a second gene.

6. A method as claimed in claim 5, wherein the mutation in a second gene is in a gene involved in the aromatic amino acid biosynthetic pathway.

7. A method as claimed in claim 6, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

8. A method as claimed in any one of the preceding claims,wherein the bacterium expresses DNA encoding a heterologous antigen.

9. A method as claimed in any one of the preceding claims, wherein the vaccine is adapted for oral administration.

10. A method of producing an attenuated Salmonella bacterium, which method comprises introducing a non-reverting mutation in the htrA gene.

11. A method as claimed in claim 10, wherein the mutation is a deletion or insertion mutation.

12. A method as claimed in claim 10 or 11, wherein the bacterium is selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

13. A method as claimed in any one of claims 10 to 12, wherein attenuation is also brought about by introducing a mutation into a second gene.

14. A method as claimed in claim 13, wherein the mutation in a second gene is in a gene in the aromatic amino acid biosynthetic pathway.

15. A method as claimed in claim 14, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

16. A method as claimed in any one of claims 10 to 15, wherein the bacterium expresses DNA encoding a heterologous antigen.

17. A vaccine comprising a pharmaceutically acceptable carrier and an effective amount of a bacterium attenuated by a non-reverting mutation in the htrA gene.

18. A vaccine as claimed in claim 17, wherein the mutation is a deletion or insertion mutation.

19. A vaccine as claimed in claim 17 or 18, wherein the bacterium is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.

20. A vaccine as claimed in claim 19, wherein the Salmonella bacterium is selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

21. A vaccine as claimed in any one of claims 17 to 20, in which attenuation of the bacterium is also brought about by a mutation in a second gene.

22. A vaccine as claimed in claim 21, wherein the mutation in a second gene is in a gene involved in the aromatic amino acid biosynthetic pathway.

23. A vaccine as claimed in claim 22, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

24. A vaccine as claimed in any one of claims 17 to 23, wherein the bacterium expresses DNA encoding a heterologous antigen.

25. A vaccine as claimed in any one of claims 17 to 24 adapted for oral administration.

26. A bacterium attenuated by a non-reverting mutation in the htrA gene for use in the prophylactic treatment of a host against infection by a microorganism.

27. A bacterium as claimed in claim 26, which is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.

28. A Salmonella bacterium attenuated by a non-reverting mutation in the htrA gene.

29. A bacterium as claimed in claim 26, 27 or 28, wherein the mutation is a deletion or insertion mutation.

30. A bacterium as claimed in any one of claims 27 to 29 selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella cholerasuis.

31. A bacterium as claimed in any one of claims 26 to 30, in which attenuation is also brought about by a mutation in a second gene.

32. A bacterium as claimed in claim 31, wherein the mutation in a second gene is in a gene in the aromatic amino acid biosynthetic pathway.

33. A bacterium as claimed in claim 32, wherein the gene involved in the aromatic amino acid biosynthetic pathway is selected from the aroC, aroA and aroD genes.

34. A bacterium as claimed in any one of claims 26 to 33 which expresses DNA encoding a heterologous antigen.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, SE

1. Impfstoff, enthaltend einen pharmazeutisch verträglichen Träger und eine wirksame Menge eines durch eine nicht-umkehrbare Mutation im **htrA**-Gen abgeschwächten Bakteriums.

2. Impfstoff nach Anspruch 1 worin die Mutation eine Deletions- oder Insertions-Mutation ist.

3. Impfstoff nach Anspruch 1 oder 2, worin das Bakterium ausgewählt ist aus der Gattung **Salmonella, Bordetella, Vibrio, Haemophilus** und **Escherichia**.

4. Impfstoff nach Anspruch 3, worin das **Salmonella**-Bakterium ausgewählt ist aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis**.

5. Impfstoff nach einem der vorhergehenden Ansprüche, worin die Abschwächung des Bakteriums ebenfalls erfolgt durch eine Mutation in einem zweiten Gen.

6. Impfstoff nach Anspruch 5, worin die Mutation eines zweiten Gens in einem Gen erfolgt, das im aromatischen Aminosäure-Biosyntheseweg involviert ist.

7. Impfstoff nach Anspruch 6, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC-, aroA-** und **aroD**-Genen.

8. Impfstoff nach einem der vorhergehenden Ansprüche, worin das Bakterium DNA exprimiert, die ein heterologes Antigen codiert.

9. Impfstoff nach einem der vorhergehenden Ansprüche, angepaßt für die orale Verabreichung.

10. Bakterium, abgeschwächt durch eine nicht-umkehrbare Mutation im **htrA**-Gen, zur Verwendung bei der prophylaktischen Behandlung eines Wirts gegen Infektion durch einen Mikroorganismus.

11. Bakterium nach Anspruch 10, ausgewählt aus der Gattung **Salmonella, Bordetella, Vibrio, Haemophilus** und **Escherichia**.

12. Salmonella-Bakterium, abgeschwächt durch eine nicht-umkehrbare Mutation im **htrA**-Gen.

13. Bakterium nach Anspruch 10, 11 oder 12, worin die Mutation eine Deletions- oder Insertions-Mutation ist.

14. Bakterium nach einem der Ansprüche 11 bis 13, ausgewählt aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis**.

15. Bakterium nach einem der Ansprüche 10 bis 14, worin die Abschwächung auch durch Mutation in einem zweiten Gen erfolgt.

16. Bakterium nach Anspruch 15, worin die Mutation in einem zweiten Gen in einem Gen im aromatischen Aminosäure-Biosyntheseweg erfolgt.

17. Bakterium nach Anspruch 16, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC-, aroA-** und **aroD**-Genen.

18. Bakterium nach einem der Ansprüche 10 bis 17, das DNA exprimiert, die für ein heterologes Antigen codiert.

Patentansprüche für folgende Vertragsstaaten : ES, GR

1. Verfahren zur Herstellung eines Impfstoffes, umfassend das Vermischen eines pharmazeutisch verträglichen Trägers und einer wirksamen Menge eines Bakteriums, abgeschwächt durch eine nicht-umkehrbare Mutation im **htrA**-Gen.

2. Verfahren nach Anspruch 1, worin die Mutation eine Deletions- oder Insertions-Mutation ist.

3. Verfahren nach Anspruch 1 oder 2, worin das Bakterium ausgewählt ist aus der Gattung **Salmonella, Bordetella, Vibrio, Haemophilus** und **Escherichia.**

4. Verfahren nach Anspruch 3, worin das Salmonella-Bakterium ausgewählt ist aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis.**

5. Verfahren nach einem der vorhergehenden Ansprüche, worin die Abschwächung des Bakteriums ebenfalls erfolgt durch eine Mutation in einem zweiten Gen.

6. Verfahren nach Anspruch 5, worin die Mutation eines zweiten Gens in einem Gen erfolgt, das im aromatischen Aminosäure-Biosyntheseweg involviert ist.

7. Verfahren nach Anspruch 6, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC-, aroA-** und **aroD**-Genen.

8. Verfahren nach einem der vorhergehenden Ansprüche, worin das Bakterium DNA exprimiert, die ein heterologes Antigen codiert.

9. Verfahren nach einem der vorhergehenden Ansprüche, angepaßt für die orale Verabreichung.

10. Verfahren zur Herstellung eines abgeschwächten **Salmonella**-Bakteriums, wobei das Verfahren die Einführung einer nicht-umkehrbaren Mutation in das **htrA**-Gen umfaßt.

11. Verfahren nach Anspruch 10, worin die Mutation eine Deletions- oder Insertions-Mutation ist.

12. Verfahren nach Anspruch 10 oder 11, worin das Bakterium ausgewählt ist aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis.**

13. Verfahren nach einem der vorhergehenden Ansprüche 10 bis 12, worin die Abschwächung ebenfalls durch Einführung einer Mutation in ein zweites Gen erfolgt.

14. Verfahren nach Anspruch 13, worin die Mutation in einem zweiten Gen in einem Gen im aromatischen Aminosäure-Biosyntheseweg erfolgt.

15. Verfahren nach Anspruch 14, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC**-, **aroA**- und **aroD**-Genen.

16. Verfahren nach einem der Ansprüche 1 bis 15, worin das Bakterium DNA exprimiert, die für ein heterologes Antigen codiert.

17. Impfstoff, umfassend einen pharmazeutisch annehmbaren Träger und eine wirksame Menge eines durch eine nicht-umkehrbare Mutation im **htrA**-Gen abgeschwächten Bakteriums.

18. Impfstoff nach Anspruch 17, worin die Mutation eine Deletions- oder Insertions-Mutation ist.

19. Impfstoff nach Anspruch 17 oder 18, worin das Bakterium ausgewählt ist aus der Gattung **Salmonella, Bordetella, Vibrio, Haemophilus** und **Escherichia.**

20. Impfstoff nach Anspruch 19, worin das **Salmonella**-Bakterium ausgewählt ist aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis.**

21. Impfstoff nach einem der Ansprüche 17 bis 20, worin die Abschwächung des Bakteriums ebenfalls erfolgt durch eine Mutation in einem zweiten Gen.

22. Impfstoff nach Anspruch 21, worin die Mutation eines zweiten Gens in einem Gen erfolgt, das im aromatischen Aminosäure-Biosyntheseweg involviert ist.

23. Impfstoff nach Anspruch 22, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC**-, **aroA**- und **aroD**-Genen.

24. Impfstoff nach einem der Ansprüche 17 bis 23, worin das Bakterium DNA exprimiert, die ein heterologes Antigen codiert.

25. Impfstoff nach einem der Ansprüche 17 bis 24, angepaßt für die orale Verabreichung.

26. Bakterium, abgeschwächt durch eine nicht-umkehrbare Mutation im **htrA**-Gen, zur Verwendung bei der prophylaktischen Behandlung eines Wirts gegen Infektion durch einen Mikroorganismus.

27. Bakterium nach Anspruch 26, ausgewählt aus der Gattung **Salmonella, Bordetella, Vibrio, Haemophilus** und **Escherichia**.

28. **Salmonella**-Bakterium, abgeschwächt durch eine nicht-umkehrbare Mutation im **htrA**-Gen.

29. Bakterium nach Anspruch 26, 27 oder 28, worin die Mutation eine Deletions- oder Insertions-Mutation ist.

30. Bakterium nach einem der Ansprüche 27 bis 29, ausgewählt aus **Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis** und **Salmonella cholerasuis**.

31. Bakterium nach einem der Ansprüche 26 bis 30, worin die Abschwächung auch durch Mutation in einem zweiten Gen erfolgt.

32. Bakterium nach Anspruch 31, worin die Mutation in einem zweiten Gen in einem Gen im aromatischen Aminosäure-Biosyntheseweg erfolgt.

33. Bakterium nach Anspruch 32, worin das im aromatischen Aminosäure-Biosyntheseweg involvierte Gen ausgewählt ist aus **aroC-, aroA**- und **aroD**-Genen.

34. Bakterium nach einem der Ansprüche 26 bis 33, das DNA exprimiert, die für ein heterologes Antigen codiert.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, SE

1. Vaccin, comprenant un véhicule pharmaceutiquement acceptable et une quantité efficace d'une bactérie atténuée par une mutation irréversible dans le gène *htrA*.

2. Vaccin selon la revendication 1, dans lequel la mutation est une mutation par délétion ou insertion.

3. Vaccin selon la revendication 1 ou 2, dans lequel la bactérie est choisie dans les genres *Salmonella, Bordetella, Vibrio, Haemophilus* et *Escherichia*.

4. Vaccin selon la revendication 3, dans lequel la bactérie appartenant au genre *Salmonella* est choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis*.

5. Vaccin selon l'une quelconque des revendications précédentes, dans lequel l'atténuation de la bactérie est également produite par une mutation dans un second gène.

6. Vaccin selon la revendication 5, dans lequel la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

7. Vaccin selon la revendication 6, dans lequel le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD*.

8. Vaccin selon l'une quelconque des revendications précédentes, dans lequel la bactérie exprime de l'ADN codant pour un antigène hétérologue.

9. Vaccin selon l'une quelconque des revendications précédentes, destiné à l'administration orale.

10. Bactérie atténuée par une mutation irréversible dans le gène *htrA*, pour utilisation dans le traitement prophylactique d'un hôte contre une infection par un micro-organisme.

11. Bactérie selon la revendication 10, qui est choisie dans les genres *Salmonella, Bordetella, Vibrio, Haemophilus* et *Escherichia*.

12. Bactérie appartenant au genre *Salmonella*, atténuée par une mutation irréversible dans le gène *htrA*.

13. Bactérie selon la revendication 10, 11 ou 12, dans laquelle la mutation est une mutation par délétion ou insertion.

14. Bactérie selon l'une quelconque des revendications 11 à 13, choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis*.

15. Bactérie selon l'une quelconque des revendications 10 à 14, dans laquelle l'atténuation est également produite par une mutation dans un second gène.

16. Bactérie selon la revendication 15, dans laquelle la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

17. Bactérie selon la revendication 16, dans laquelle le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD*.

18. Bactérie selon l'une quelconque des revendications 10 à 17, qui exprime de l'ADN codant pour un antigène hétérologue.

Revendications pour les Etats contractants suivants : ES GR

1. Procédé de production d'un vaccin, comprenant le mélange d'un véhicule pharmaceutiquement acceptable et d'une quantité efficace d'une bactérie atténuée par une mutation irréversible dans le gène *htrA*.

2. Procédé selon la revendication 1, dans lequel la mutation est une mutation par délétion ou insertion.

3. Procédé selon la revendication 1 ou 2, dans lequel la bactérie est choisie dans les genres *Salmonella, Bordetella, Vibrio, Haemophilus* et *Escherichia*.

4. Procédé selon la revendication 3, dans lequel la bactérie appartenant au genre *Salmonella* est choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis*.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'atténuation de la bactérie est également produite par une mutation dans un second gène.

6. Procédé selon la revendication 5, dans lequel la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

7. Procédé selon la revendication 6, dans lequel le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD*.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel la bactérie exprime de l'ADN codant pour un antigène hétérologue.

9. Procédé selon l'une quelconque des revendications précédentes, dans lequel le vaccin est destiné à l'administration orale.

10. Procédé de production d'une bactérie atténuée appartenant au genre *Salmonella*, lequel procédé comprend l'introduction d'une mutation irréversible dans le gène *htrA*.

11. Procédé selon la revendication 10, dans lequel la mutation est une mutation par délétion ou insertion.

12. Procédé selon la revendication 10 ou 11, dans lequel la bactérie est choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis*.

13. Procédé selon l'une quelconque des revendications 10 à 12, dans lequel l'atténuation de la bactérie est également produite par introduction d'une mutation dans un second gène.

14. Procédé selon la revendication 13, dans lequel la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

15. Procédé selon la revendication 14, dans lequel le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD*.

16. Procédé selon l'une quelconque des revendications 10 à 14, dans lequel la bactérie exprime de l'ADN codant pour un antigène hétérologue.

17. Vaccin, comprenant un véhicule pharmaceutiquement acceptable et une quantité efficace d'une bactérie atténuée par une mutation irréversible dans le gène *htrA*.

18. Vaccin selon la revendication 17, dans lequel la mutation est une mutation par délétion ou insertion.

19. Vaccin selon la revendication 17 ou 18, dans lequel la bactérie est choisie dans les genres *Salmonella, Bordetella, Vibrio, Haemophilus* et *Escherichia*.

20. Vaccin selon la revendication 19, dans lequel la bactérie appartenant au genre *Salmonella* est choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis*.

21. Vaccin selon l'une quelconque des revendications 17 à 20, dans lequel l'atténuation de la bactérie est également produite par une mutation dans un second gène.

22. Vaccin selon la revendication 21, dans lequel la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

23. Vaccin selon la revendication 22, dans lequel le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD.*

24. Vaccin selon l'une quelconque des revendications 17 à 23, dans lequel la bactérie exprime de l'ADN codant pour un antigène hétérologue.

25. Vaccin selon l'une quelconque des revendications 17 à 24, destiné à l'administration orale.

26. Bactérie atténuée par une mutation irréversible dans le gène *htrA,* pour utilisation dans le traitement prophylactique d'un hôte contre une infection par un micro-organisme.

27. Bactérie selon la revendication 26, qui est choisie dans les genres *Salmonella, Bordetella, Vibrio, Haemophilus* et *Escherichia.*

28. Bactérie appartenant au genre *Salmonella,* atténuée par une mutation irréversible dans le gène *htrA.*

29. Bactérie selon la revendication 26, 27 ou 28, dans laquelle la mutation est une mutation par délétion ou insertion.

30. Bactérie selon l'une quelconque des revendications 27 à 29, choisie parmi *Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis* et *Salmonella cholerasuis.*

31. Bactérie selon l'une quelconque des revendications 26 à 30, dans laquelle l'atténuation est également produite par une mutation dans un second gène.

32. Bactérie selon la revendication 31, dans laquelle la mutation dans un second gène est dans un gène impliqué dans la voie de biosynthèse des aminoacides aromatiques.

33. Bactérie selon la revendication 32, dans laquelle le gène impliqué dans la voie de biosynthèse des aminoacides aromatiques est choisi parmi les gènes *aroC, aroA* et *aroD.*

34. Bactérie selon l'une quelconque des revendications 26 à 33, qui exprime de l'ADN codant pour un antigène hétérologue.

Fig. 1.

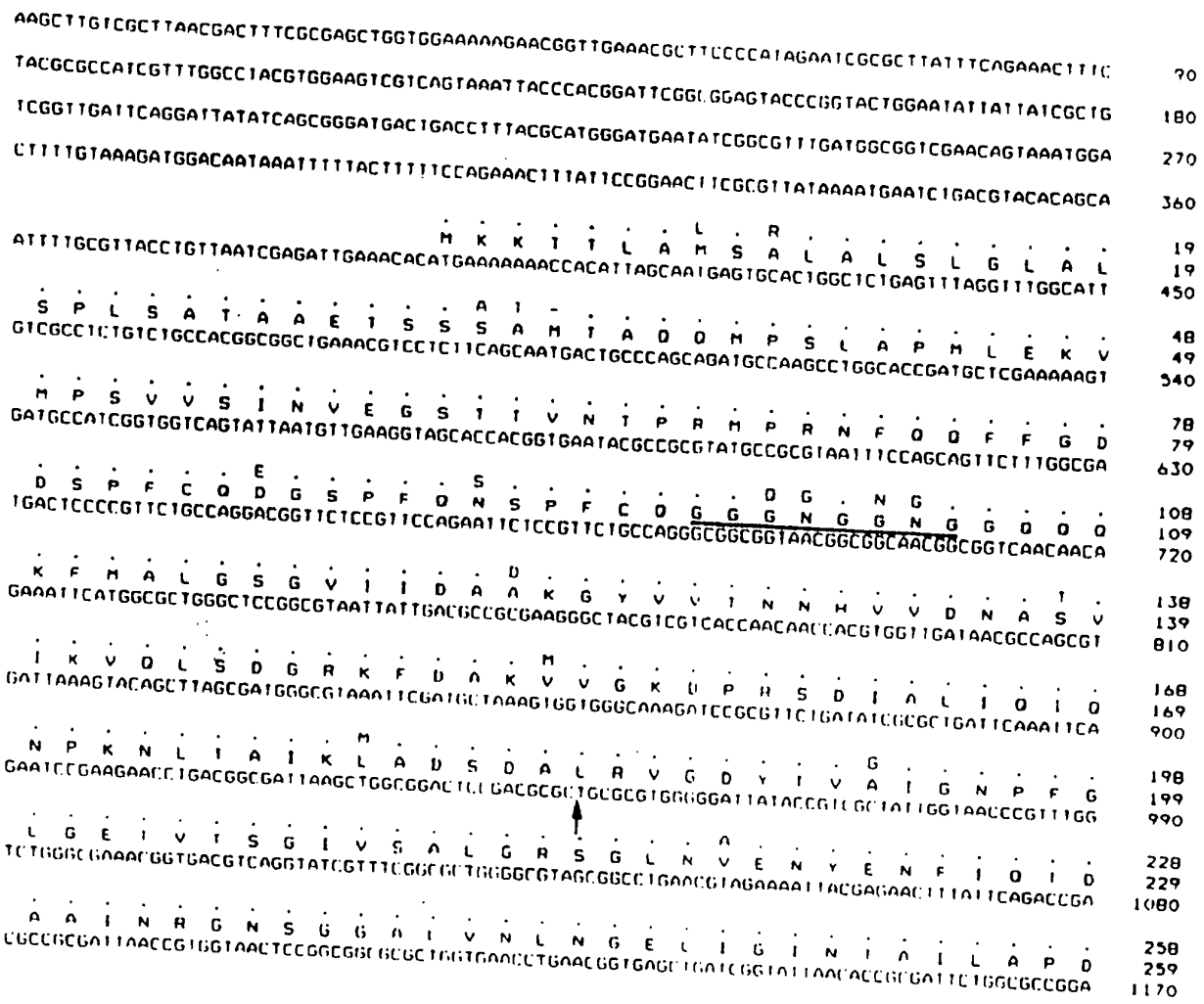
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Fig. 1(cont.)

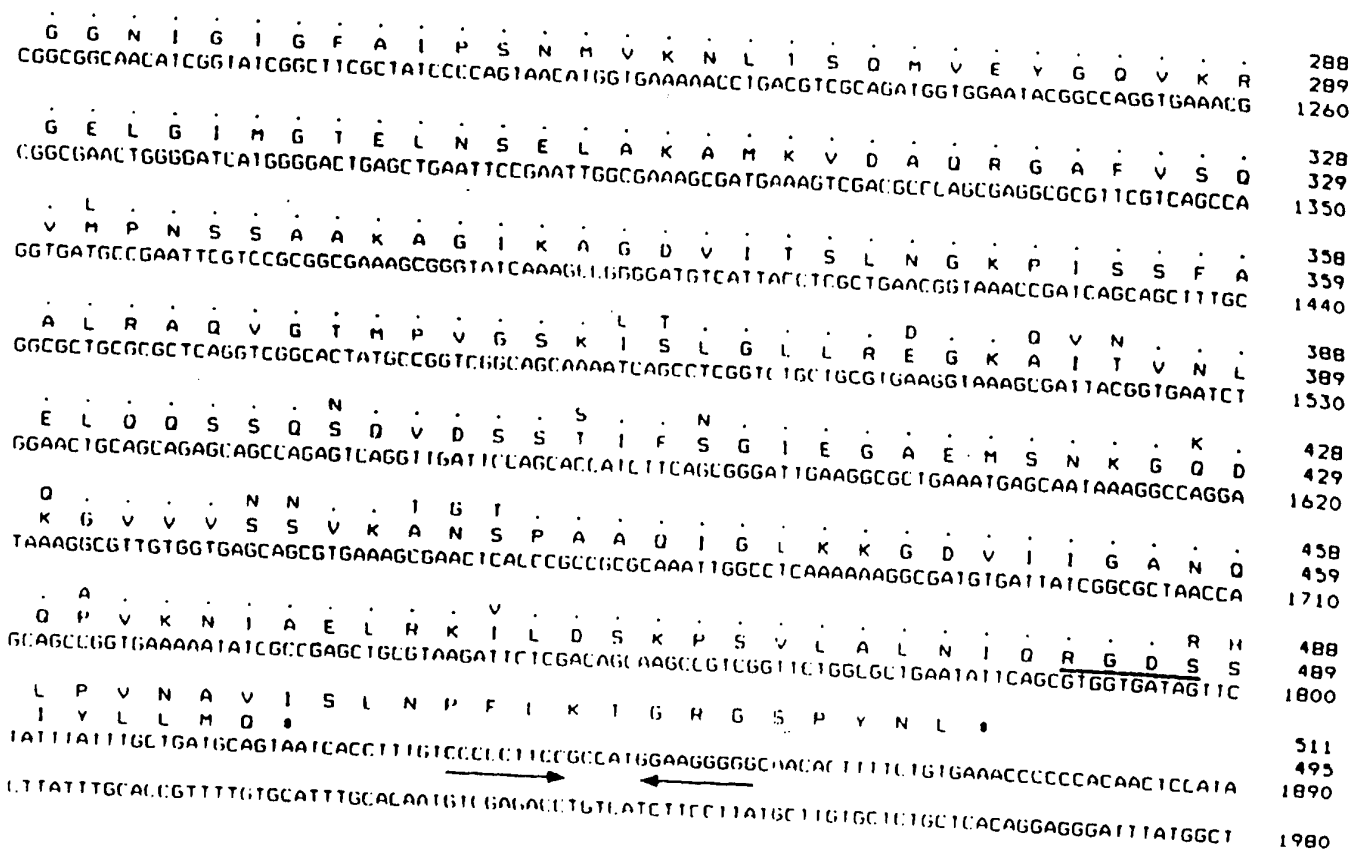
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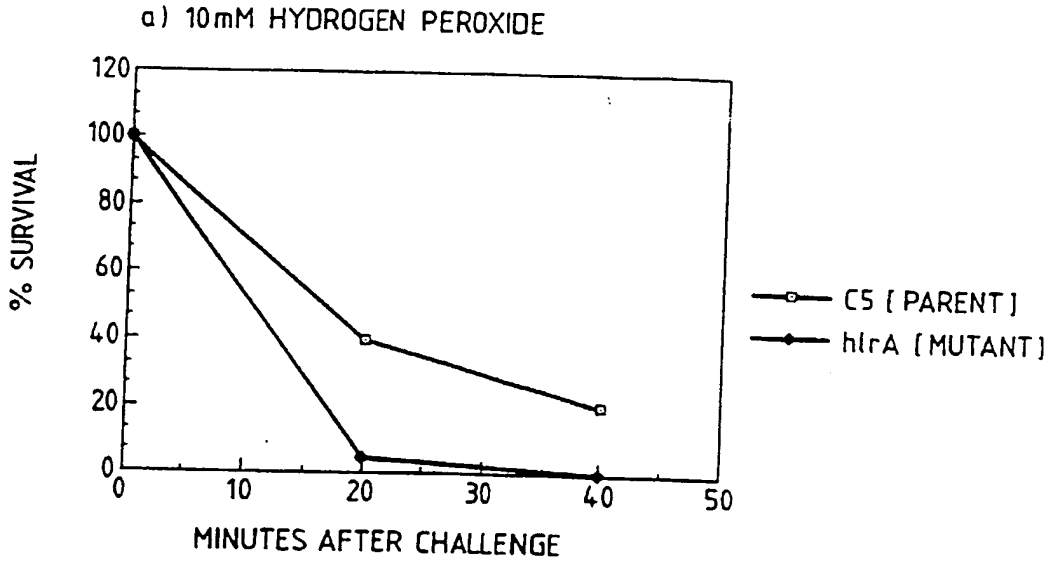
Fig.2.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% SURVIVAL
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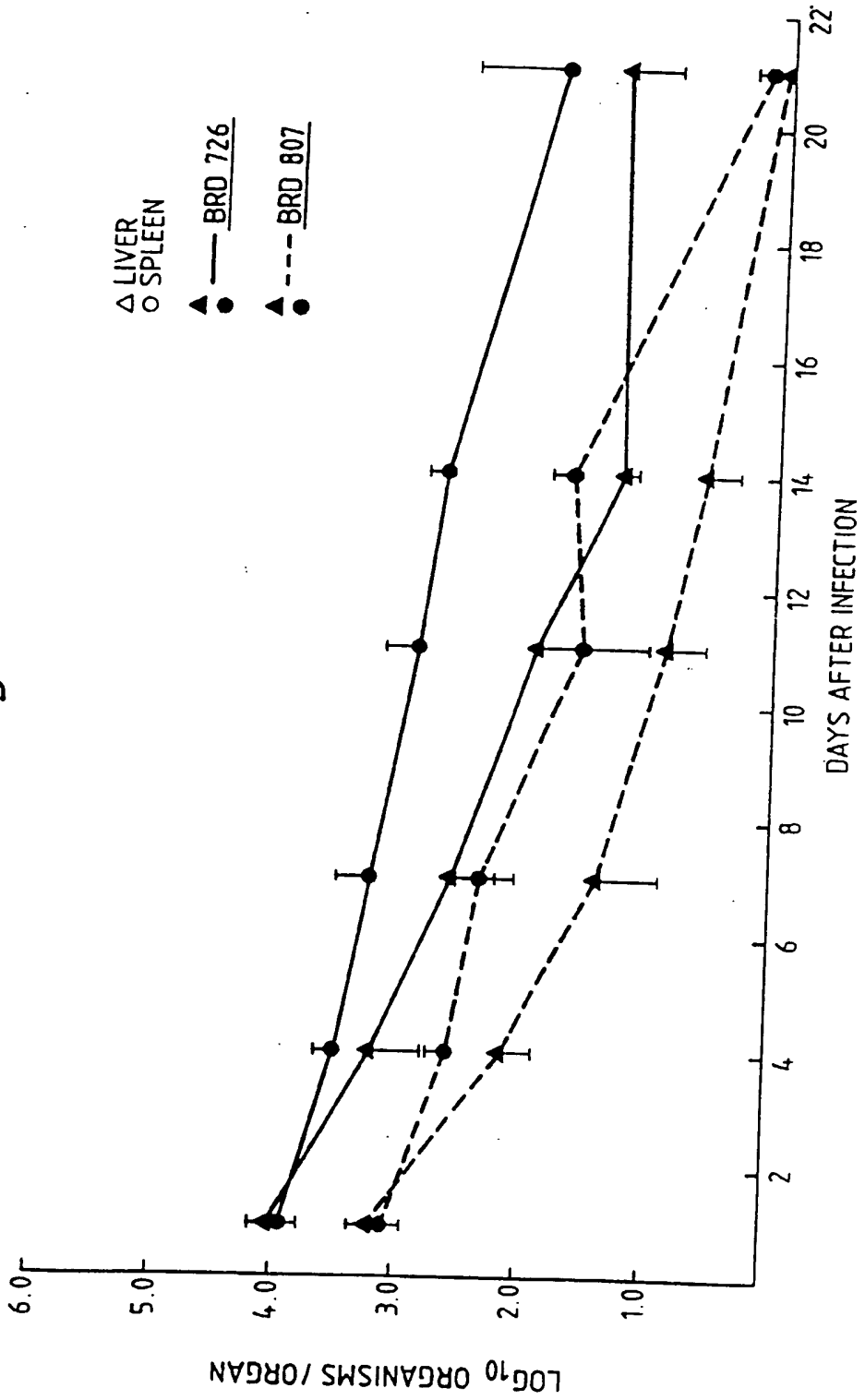
100
80
60
40
20
0
0
10
20
30
40
50
MINUTES AFTER CHALLENGE
C5 [PARENT]
hlrA [MUTANT]

Fig.3.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○ SPLEEN
BRD 726
BRD 807
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4.0
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1.0
2
4
6
8
10
12
14
16
18
20
22
DAYS AFTER INFECTION